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Effects of cereal seed protinaceous extracts on α-amylase and proteinase activity of salivary glands of Carob moth, *Ectomyelois ceratoniae* (Lepidoptera: pyralidae)

Ehsan Borzoui, Ali Reza Bandani* and Seyed Hossein Goldansaz

Plant Protection Department, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.

Abstract: The carob moth is one of the most devastating pests of pomegranate and some other products. Various pest control measures have been undertaken in order to control this pest but none of them has been successful so far. In the current study the effects of cereal seed proteinaceous extracts including triticale and three wheat cultivars (MV17, Aflak, and Zare) have been studied on α-amylase and protease activity of salivary glands of this insect.Initial screening showed 38, 44, 28 and 76% inhibitory effect for triticlae, MV-17, Aflak, and Zare cereal seed extracts respectively on α-amylase activity. Further studies were performed with Zare wheat cultivar using various concentrations including 13, 6.5, 3.25, 1.625 and 0.8125 µg protein on the enzyme activity and results showed that they inhibited the enzyme activity by 76, 75, 68, 60, and 42%, respectively. Gel assays confirmed the spectrophotometric data i.e the effect of the seed extract on the enzyme was dose dependant. The same trend was observed when seed extracts were tested against proteinase activity. These data suggest that plants produce different proteins with different specificity toward herbivores digestive enzymes some of which could be used for insect control in IPM program.

Keywords: Carob moth; α-amylase; proteinase; cereal seed extract

Introduction

The pomegranate, *Punica granatum* (Punicaceae), is one of the oldest cultivated species which is native to Iran but nowadays is widespread throughout the Mediterranean area of Asia, Africa and Europe (Durgac *et al.,* 2008). The carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is one of the most important pests of a wide range of products including dates, almonds, carob, pomegranate, nuts, walnuts, figs, pistachios, citrus, etc. (Dhouibi and Abderahmane, 2002; Nay and Perring, 2006; Mozaffarian *et al.,* 2007). The larvae feed inside the fruit and cause a great damage to fruit quality (Shakeri, 1993) such that in some areas they spoil more than 80% of the fruits (Shakeri, 2004). Various agricultural and mechanical methods including removing the flags, covering pomegranate fruit neck by straw, collecting and destroying of the infested fruit during the winter season as well as biological control are listed for the pest control (Kishani *et al.,* 2012; Nasrollahi *et al.,* 1998). However, these methods cannot control the insect pest properly. Also, since the larvae feed inside the fruit, pesticide applications in order to control this insect were not successful because the insects are not exposed to the insecticides. In addition, the public and academia concerns over the use of pesticides on fruits and food have raised many issues. Moreover, the

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* **Corresponding author**, e-mail: abandani@ut.ac.ir
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occurrence of resistance in the insect pests is another issue which strengthens the need for alternative pest control strategies in IPM (integrated pest management) (Hagstrum and Subramanyam, 1996; Yildirim *et al.,* 2001; Isman, 2006). In recent years considerable investigations have been done on plant and microbe derived materials for potentially useful products and genes to be used in pest control. Many plants have received gene/s encoding toxic proteins as a strategy to resist or be protected from insect pests and pathogens. Genes that their products are toxic to insect species are lectins (Gatehouse *et al.,* 1997), α-amylase inhibitors (Morton *et al.,* 2000), protease inhibitors (De Leo *et al.,* 2001; Falco and Silva-Filho 2003; Alfonso-Rubi *et al.,* 2003), toxins from *Bacillus thuriengiensis* (Bt toxins) (Sharma and Ortiz, 2000), and even fusion proteins consisting of plant lectin, *Galanthus nivalis* agglutinin (GNA) linked to toxic peptide (Fitches *et al.,* 2004; Down *et al.,* 2006; Fitches *et al.,* 2009).

Digestive enzymes especially α-amylase and protease are potentially a good target for insect control using inhibitors present in the plant seeds. Cereal and legume seeds are rich sources of insect digestive enzyme inhibitors. Thus, one important aspect of the insect pest control is to materialize selective inhibition of the digestive enzymes in order to interfere with digestion process of the insect by producing detrimental effects on larval and insect growth by inhibition of the digestion and assimilation of nutrients. Therefore, in order to achieve a control strategy based on digestive enzyme inhibitors it is advisable to characterize digestive enzymes as well as to do *in vitro* and *in vivo* bioassays with plant proteinaceous inhibitors (Horrison and Bonning, 2010).

A first example of introduction of plant genes encoding toxic protein against insect is cowpea trypsin inhibitor which is expressed in tobacco leaves to combat lepidopteran larvae (Hilder *et al.,* 1987, Silva *et al.,* 2001). Since then, attempts have been made to use toxic plant proteins against insects with some success. α-Amylase inhibitor gene form seeds of common bean (*Phaseolus vulgaris*) when transferred to pea confer resistance to pea weevil (*Bruchus pisorum*) (Morton *et al.,* 2000; Silva *et al.,* 2001). Also, transformed Azuki bean (*Vigna angularis*) confers resistance to *Callosobruchus chinensis* and *C. maculatus* (Ishimoto *et al.,* 1996; Silva *et al.,* 2001).

So far, no investigation has been done on the carob moth salivary gland enzymes. It has long been established that salivary glands of the caterpillar have glucose oxidase (GOX) activity (Markx-Jacques and Bede, 2005; Peiffer and Felton, 2005; Babic *et al.,* 2008). Our hypothesis was that caterpillars produce labial GOX in order to oxidize glucose to gluconate, a nutritionally unavailable carbohydrate. Thus, saliva should have carbohydrate digesting enzymes including α-amylases. One strategy to cope with excess carbohydrate in the caterpillar diet is to produce salivary enzymes that convert carbohydrate to glucose so that GOX has substrate to act upon and as a result to prevent glucose absorption. Thus, the aim of the current study was to make in depth analysis of the salivary gland enzymes using spectrophotometric and in gel assays procedures. Also, the effects of cereal seed inhibitors including triticale and three cultivars of wheat on the insect salivary enzymes were investigated.

Materials and Methods

Insect rearing

A population of *E. ceratoniae* was collected from Chandab Region,Varamin, Tehran province, Iran. The larvae were reared on artificial diet under laboratory conditions as described by Norouzi *et al.,* (2008) with slight modification such as rearing at 29.6 ± 5 °C, with a 16:8 h photoperiod and 75 ± 5% RH in contrast to Norouzi *et al.,* (2008) who reared at 30 ± 1 °C and 70 ± 5% RH.

Enzymes preparation

Salivary glands (SGs) were removed and the enzyme preparation was done based on Kazzazi *et al.,* (2005). The larvae were carefully dissected in cooled distilled water under stereomicroscope (Stemi SV6 ZEISS,

Germany). SGs were separated and homogenized in pre-cooled homogenizer (Teflon pestle). The homogenates were centrifuged at 15,000g for 15 min at 4 °C. The supernatants were pooled and stored at -20 °C as an enzyme source for subsequent analyses.

Optimum pH for salivary gland enzymes activity

The optimum pH for the activities of the salivary gland α-amylase and protease enzymes was determined using universal buffer (0.02 M) (Hosseinkhani and Nemat-Gorgani, 2003) with pH set at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0.

α-Amylase assay

α-Amylase activity of SG was assayed by the dinitrosalicilic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch solution as substrate as described by Bandani *et al.,* (2009). One unit of α-amylase activity was defined as the amount of enzyme required to produce 1mg maltose in 30 min at 35 ^{o}C. A standard curve of absorbance against amount of maltose released was constructed to enable calculation of the amount of maltose released during α-amylase assay. A blank without substrate but with α-amylase extract and a control containing no α-amylase extract with substrate were run simultaneously with reaction mixture. All assays were performed in triplicates and with three replications.

Protease assay

General proteolysis assay was done according to the methods of Elpidina *et al.,* (2001) and Gatehouse *et al.,* (1999), with slight modification. Briefly, 10 µl enzyme extract and 50µl substrate solution (Azocasein 2%) were mixed with 40 µl 20 mM Glycine-NaOH buffer (pH 10). After 60 min incubation, 100 µl 30% trichloroacetic acid (TCA) was added to the reaction mixture, and kept at 4 °C for 30 min, followed by centrifugation at 15,000g for 15 min to precipitate non hydrolysed substrate. 100µl NaOH (1 M) was added to 100 µl supernatant and the absorbance at 405 nm was measured.

Seed protein extraction

Seed proteins were extracted according to Baker (1987) and Melo *et al.,* (1999). Seeds were powdered thoroughly, and then 30 grams of powdered seeds from each plant separately was mixed with a solution of 0.1 M NaCl and stirred for 3 h, followed by centrifugation at 8,000g for 30 min. The pellet was discarded, and the supernatant was placed at 70 °C for 20 min in order to inactivate enzymes within the seeds. Proteins were concentrated using a saturation of 70% ammonium sulfate followed by centrifugation at 8,000g for 30 min at 4 °C. The pellet was dissolved in ice-cold sodium phosphate buffer (0.02 M and pH 7.0) and was dialyzed against the same buffer for 20 h. This dialyzed solution was used as inhibitors in enzymatic assay tests.

The effect of inhibitor on alpha-amylase activity

The effects of the seed extracts on the amylase activity were determined as described by Mehrabadi *et al.,* (2010). Enzyme extract was pre-incubated with Triticale and three cultivars of wheat seed extracts for 30 min at 35 °C followed by determination of the enzyme activity as described before using dinitrosalicilic acid (DNS) procedures. Appropriate blanks were included in the experiments as well. The inhibition percentage (% I) was calculated by the method of Mehrabadi *et al.,* (2011):

%I = 100 X [(A540 control – A540 Exp) / A540 control].

The effect of inhibitor on general protease activity

The effects of the seed extracts on general protease activity were determined as described by Saadati *et al.,* (2011). Enzyme was pre-incubated with Triticale and three cultivars of wheat seed extracts for 30 min at 35 °C followed by determination of the enzyme activity as described before. The inhibition percentage was calculated as described for amylase.

In gel assay

Electrophoretic detection of proteolytic enzymes was done basically according to the procedures described by Laemmli (1970) and Walker *et al.,* (1998). PAGE was performed in 10% (w/v) gel co-polymerized with 0.1% gelatin and 4% for stacking gel with 0.05% SDS. Electrophoresis was conducted at 4 ^{o}C until the blue dye reached the bottom of the gel. Then, the gel was rinsed with distilled water and washed by 2.5% (v/v) Triton X-100 buffer for 60 min followed by incubation in Glycine-NaOH buffer (pH 10) for about 6 h. Finally, the gel was stained as described by Saadati *et al.,* (2011). Amylolytic activity in the gel was detected using procedures described by Mehrabadi *et al.,* (2011). Briefly, PAGE was performed in 10% (w/v) gel for separating gel and 5% for stacking gel with 0.05% SDS. Electrophoresis was conducted at a voltage of 120V until the blue dye reached the bottom of the gel. The gel was rinsed with distilled water and washed by 1% (v/v) Triton X-100 buffer for 15 min. Then, the gel was incubated in glycine-NaOH buffer (pH 9) containing 1% starch solution, 2 mM $CaCl_2$ and 10 mM NaCl for 1.5 h. Finally, the gel treated with a solution of 1.3% I_2 and 3% KI to stop the reaction and to stain the un-reacted starch background. Zones of α-amylase activities appeared as light band against dark background.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin as a standard.

Statistical anaylysis

Data were analysed based on a completely randomized design using SAS software. Mean comparison was done using Duncan's test.

Results

α-Amylase activity and effect of pH on the α-amylase activity

Using dinitrosalicilic acid procedure it was found that α-amylase was active in the moth salivary glands. The α-amylase was active on a wide range of pH from 5.0 to 11.0 with an optimum activity at pH 9.0. The α-amylase activity level in optimal pH was 0.0041 µmol/min/mg protein (Fig. 1).

Protease activity and effect of pH on the protease activity

To detect protease activity in the salivary gland extract, azocasein (2%) was used as a substrate. Salivary protease activity was low in acidic pH but optimum pH for protease activity was 10.0 (Fig. 1). The protease activity level in optimal pH was 0.000846 U/min/mg protein.

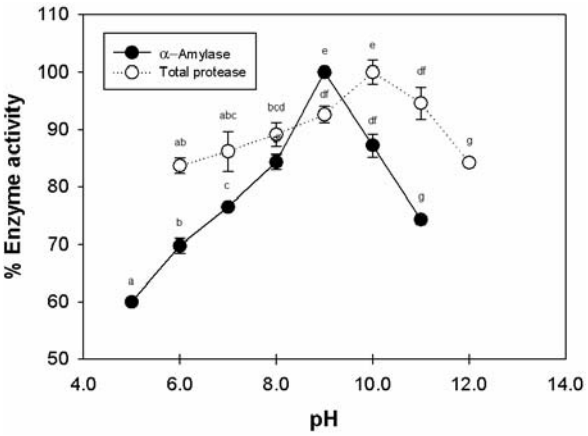

Figure 1 Effect of pH on α-amylase and total protease activity extracted from salivary gland of carob moth.

Effect of the seed extract on α-amylase

Four plant proteinaceous extracts from triticale and three wheat cultivars were tested against α-amylase and protease of the moth. To choose suitable concentration, preliminary assay was done so that based on these studies 13 µg protein was chosen to start enzyme assay. As seen in Figure 3, the effect of these seed extracts (13 µg protein) on the enzyme activity varied among triticale and wheat cultivars. Inhibitory effects of the extracts on α-amylase activity were 38, 44, 28 and 76% for triticlae, MV-17, Aflak, and Zare, respectively. Protein extracted from Zare wheat cultivar had the greatest inhibitory activity and inhibited the enzyme activity about 76% (Fig. 2). So, further studies were performed with Zare wheat

cultivar using various concentrations of 13, 6.5, 3.25, 1.625 and 0.8125 µg protein which inhibited the enzyme activity by 76, 75, 68, 60, and 42%, respectively (Fig. 2).

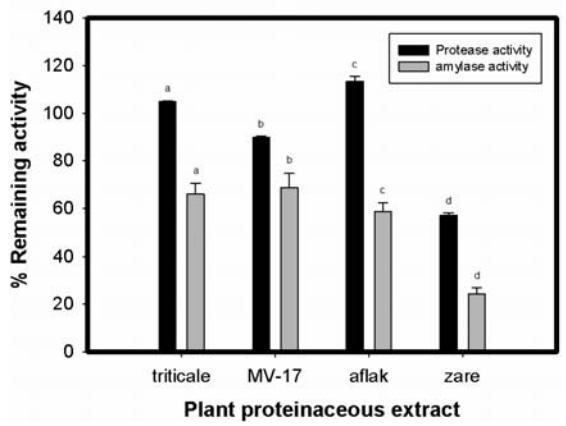

Figure 2 Inhibition of carob moth α-amylase and total protease by 13 µg protein of proteinaceous seed extract of tritcale and three cultivars of wheat. Each column is an average of three replications.

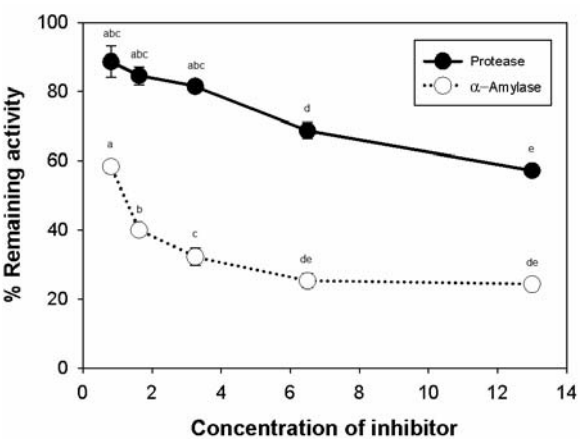

Figure 3 Inhibition of carob moth α-amylase and total protease activity by different concentrations of Zare cultivar including 13, 6.5, 3.25, 1.625 and 0.8125 µg protein of the seed extract. Enzyme and inhibitor were pre-incubated for 30 min prior to the addition of starch to measure enzyme activity. Each point represents an average of three measurements.

Protease inhibition assay

The effect of triticale and the three cultivars of wheat seed extracts at concentration of 13 µg protein were studied on protease activity of the salivary glands of the Carob moth. The effect of the seed extracts on the enzyme varied based on the type of the cultivar. The Zare cultivar seed extract had the greatest effect on protease activity (43% inhibition) followed by MV-17, triticale, and Aflak which inhibited protease activity by 11, 0, and 0%, respectively (Fig. 2). Seed extracts of triticale and aflak cultivars at concentration of 13 µg did not produce any significant effect on protease activity. Further study was done using different concentrations of Zare cultivar including 13, 6.5, 3.25, 1.625 and 0.8125 µg protein of the seed extract which produced 43, 32, 19, 16, and 12% protease inhibition, respectively (Fig. 3). Almost 43% of the enzyme inhibition was achieved when the highest concentration (13 µg protein) of the seed extract was used and 12% of the enzyme activity was produced when the lowest concentration (0.8125 µg protein) of the seed extract was used (Fig. 3).

In-gel α-amylase and protease assays

Using gel assays, it was found that there are two major bands in the salivary glands of carob moth with alpha-amylase activity (Fig. 4a). However, when different concentrations of the seed extracts (Zare cultivar of wheat) were used, varied percentages of inhibition were achieved. So, as the seed extract dose was reduced the intensity of the bands decreased. Indeed, a dose dependant manner of alpha amylase inhibition was achieved so that at high dose the amount of inhibition was more than when low dose of proteinaceous seed extract was used. As seen in Figure 4, at low dose (0.8125µg protein) intensity of the two amylase bands were slightly decreased (Fig. 4b) but at high dose (13 µg protein) the two bands almost disappeared (Fig. 4f). To detect protease activity, one-dimensional zymogram of a gelatin-containing acrylamide gel was used (Fig. 5). There were four protease bands in the moth salivary glands. Different concentrations of the seed extract of Zare cultivar i.e. 6.5, 3.25, 1.625 and 0.8125 µg protein, did not significantly change the intensity of the bands. However, at

concentration of 13 µg protein of the seed extract the intensity of the protease band was reduced (Fig. 5).

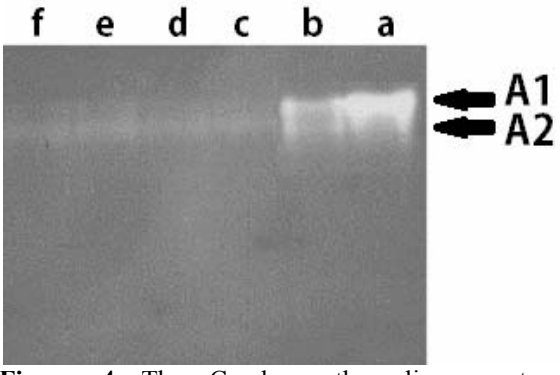

Figure 4 The Carob moth salivary extract zymogram using 1% starch as substrate. Enzyme extract was pre-incubated with different concentrations of Zare wheat cultivar for 30 min and then gel was run at 4 °C. The gel was stained with a solution of 1.3% I2 and 3% KI. Lane numbers are as follows: (a) Salivary gland extract with no inhibitor, (b) 0. 0.8125 µg.ml-1, (c) 1.625 µg.ml-1, (d) 3.25 µg.ml-1, (e) 6.5 µg.ml-1, (f) 13 µg.ml-1 protein of seed extract.
A1: α-amylase first isoenzyme
A2: α-amylase second isoenzyme

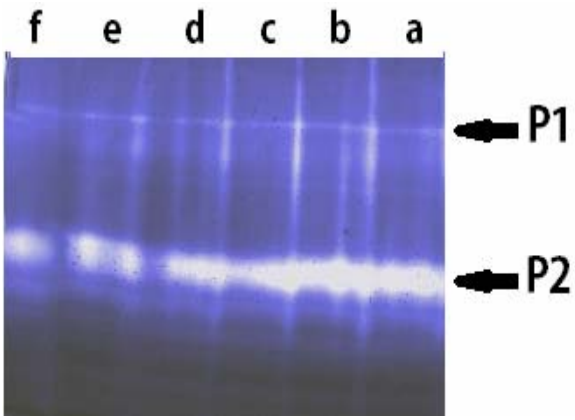

Figure 5 The Carob moth salivary extract zymogram using 0.1% gelatin as substrate. Enzyme extract was pre-incubated with different concentrations of Zare wheat cultivar for 30 min and then gel was run at 4 °C. Lane numbers are as follows: (a) Salivary gland extract with no inhibitor, (b) 0. 0.8125 µg.ml-1, (c) 1.625 µg.ml-1, (d) 3.25 µg.ml-1, (e) 6.5 µg.ml-1, (f) 13 µg.ml-1 protein of seed extract.

Discussion

In this study for the first time it was shown that the carob moth salivary gland contains enzyme activity including α-amylase and protease which so far has not been reported from this insect though other studies indicated presence of different enzymes in caterpillar oral secretions including α- and β- glucosidases and glucose oxidase (Eichenseer *et al.,* 1999; Musser *et al.,* 2002; Merkx-Jacques and Bede, 2004 and 2005; Peiffer and Felton, 2005; Babic *et al.,* 2008). Caterpillars secrete saliva from labial and mandibular glands which assists the insect in their feeding process (Elzinga, 1987). These secretions mix with plant materials and assist the ground plant materials to be transported into oral cavity. In addition so far enzymes including GOX (glucose oxidase) that catalyze the oxidation of glucose thus forming gluconate and hydrogen peroxide (H_2O_2); ascorbate peroxidase, which catalyzes the oxidation of vitamin C and concomitant reduction of H_2O_2; superoxide dismutase (SOD). SOD catalyzes the removal of highly cytotoxic superoxide radicals producing less toxic hydrogen peroxide, and catalase, an enzyme that catalyzes the degradation of H_2O_2 have been identified in the caterpillar saliva (Madhusudan *et al.,* 1994; Matthews *et al.,* 1997; Eichenseer *et al.,* 1999; Ni *et al.,* 2000; Merkx-Jacques and Bede, 2004; Babic *et al.,* 2008).

GOX is the predominant enzyme in the labial salivary glands of caterpillars thus it is hypothesized that insect produces labial GOX in order to oxidize glucose to hydrogen peroxide and gluconate. Gluconate is not absorbed by the insect (Merkx-Jacques and Bede, 2004 and 2005; Peiffer and Felton, 2005; Babic *et al.,* 2008). Thus, insect saliva should contain enzyme/s such as α-amylase in order to act on carbohydrate to produce glucose. It has been established that insect diet influences GOX activity since it was shown that *Spodoptera exigua* caterpillar reared on artificial diet had more (ten times more) GOX activity than those fed on plants (Merkx-Jacques and Bede, 2004). Thus, one strategy for caterpillar to cope with excess carbohydrate is

to produce salivary enzymes that convert carbohydrate to glucose so that GOX have substrate to act upon carbohydrate entering gut and make them unavailable for insect.

This study showed that two isoenzymes of the α-amylase are present in the salivary gland. Presence of different isoenzymes in insects shows their importance in insect's digestion of carbohydrate to guarantee effective digestion (Mehrabadi *et al.,* 2012). There are reports that many insect species, which rely on carbohydrate metabolism for their survival, have more than one isoenzyme α-amylase excreted by digestive tissues (Mehrabadi *et al.,* 2010). Indeed, the presence of a number of α-amylase isoenzymes is a strategy to escape from plant secondary metabolites (Silva *et al.,* 1999). Production of α-amylase was also detected in other insect species including *Sitophilus zeamais* Motschulsky, *Callosobruchus maculatus* Fabricius, *Zabrotes subfasciatus* Boheman, *Acanthoscelides obtectus* Say, and *Eurygaster integriceps* Puton (Silva *et al.,* 1999; Franco *et al.,* 2005; Bandani *et al.,* 2009).

The carob moth α-amylase and protease activities were the highest at alkaline pH. This is because after the secretion of the enzyme by salivary gland, it is mixed with ingested food and enters the alimentary canal where pH is alkaline and that could be one reason that the salivary gland enzymes are adapted to function better at alkaline pH. There are repots that caterpillar gut pH is alkaline and their digestive enzymes are active in alkaline pH, too. Although α-Amylase in salivary gland was shown to be active on a wide range from pH 5.0 to 11.0, the optimum pH was 9.0. Optimum pH of carob moth was similar to that of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) (pH 9.0), *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) (pH 10.0) and *Naranga aenescens* Moore (pH 9.0) (Zibaee et al, 2008; Yezdani *et al.,* 2011; Asadi et al, 2010). Some others such as hemipteran insects enzymes are active at acidic pH e.g. *Lygus hesperus*, *L. lineolaris* and *Eurygaster integriceps* and *E. maura* (Zeng and Cohen, 2000; Ravan *et al.,* 2009).

Since the insects such as carob moth are somehow dependent on α-amylases for their survival, these enzymes could be a good target for insect control through α-amylase inhibitors (Franco *et al.,* 2002; Svensson *et al.,* 2003; Sivakumar *et al.,* 2006). Moreover, there are many repots that enzyme inhibitors are abundant in cereals and legumes (Franco *et al.,* 2002; Sivakumar *et al.,* 2006). These molecules play an important role in plant defense against pests and pathogens. Bannakan *et al.,* (2007) showed that mungbean seed extract impaired *Callosobruchus maculatus* larval development. Also, they found significant inhibition (up to 100%) of α-amylase of the insect *in vitro*. Ishimoto and Kitamura (1988) found that α-amylase inhibitor present in the pea seeds is the main resistance factor to *Bruchus pisorum* Linnaeus, *C. maculatus* Fabricius, and *C. chinensis* Linnaeus.

In this study it was found that different cultivars of wheat have different specificity toward α-amylases of carob moth. The least activity was seen for triticale seed extract with 38% inhibition of the enzyme activity of carob moth and the greatest activity was seen for wheat cv. Zare seed extract with 76% inhibition of the enzyme activity. Mehrabadi *et al.,* (2010) showed that triticale seed extract inhibited the Sunn pest (*Eurygaster integriceps*) α-amylase activity around 80%. These data indicate that proteinaceous extract of a specific seed shows different specificity toward different insect species which is an important step in developing molecules for production of insect resistant transgenic plants (Valencia *et al.,* 2000).

Also, it was found that different wheat cultivars have different effects on enzyme activity i.e. cultivars MV-17, Aflak and Zare inhibited carob moth enzyme activity 44, 28 and 76%, respectively. Further study showed that the effect was dose dependent i.e. when 13 µg protein was used 76% enzyme inhibition was achieved but at low doses (0.81 µg protein) only 42% of carob α-amylase was inhibited. Almost the same trend of α-amylase inhibition was obtained against proteinase i.e. lower protease inhibition was achieved when tiriticale, Aflak and MV-17 seed extracts were used and the greatest protease inhibition was achieved for Zare cultivar. Also,

the effect of Zare seed extract on the protease activity was dose dependent. Overal, our data is in agreement with the findings of Valencia *et al.*, (2000) who showed a dose dependent inhibition of *Amaranthus cruentus* Linnaeus against α-maylase of coffee berry borer, *Hypothenemus hampei* Ferrari. Khan (2011) reported proteinaceous inhibitors isolated from Chick pea (*Cicer arietinum*), Kidney bean (*Phaseolus vulgaris*), Maize seeds (*Zea mays*), Wheat (*Triticum aestivum*) and Millet (*Pennisetum typhoides*) exhibited inhibitory activity against α-amylase from red flour beetle, *Tribolium castaneum*. It was reported that wheat, maize and kidney bean inhibitors inhibited the insect (*T. Castaneum*) α-amylase by 75, 82, and 67.8%, respectively (Khan, 2011).

In conclusion it could be said that different plant species produce different proteins with different specificity which can be used in plant protection strategies. Specificity of inhibitors is an important issue since the introduced inhibitors must not adversely affect the plant's own enzymes (α-maylase) as well as mammals' enzymes.

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References

Alfonso-Rubi, J., Ortego, F., Castariera, P., Carbonero, P. and Diaz, I. 2003. Transgenic expression of trypsin inhibitor Cme from barley in indica and japonica rice, confers resistance to the rice weevil Sitophilus oryzae. Transgenic Research, 12: 23-31.

Asadi, A., Ghadamyari, M., Sajedi, R. H., sendi, J. J. and Tabari, M. 2010. Biochemical characterization of midgut, salivary glands and haemolymph α-amylases of *Naranga aenescens*. Bulletin of Insectology, 63: 175-181.

Babic, B., Poisson, A., Darwish, S., Lacasse, J., Merkx-Jacques, M., Despland, E. and Bede, J. C., 2008. Influence of dietary nutritional composition on caterpillar salivary enzyme activity. Journal of Insect Physiology, 54: 286-296.

Baker, J. E. 1987. Purification of isoamylases from the rice weevil, *Sitophilus orizae* L. by HPLC and their interaction with partially purified amylase inhibitor from wheat. Insect Biochemistry, 17: 37-44.

Bandani, A. R., Kazzazi, M. and Mehrabadi, M. 2009. Purification and characterization of midgut α-Amylase of *Eurygaster integriceps*. Entomological Science, 12: 25-32.

Bannakan, I., Hormchan, P., Wongpiyasatid, A. and Engkakul, A. 2007. Effects of α-amylase Inhibitor on Mungbean Weevil, *Callosobruchus maculatus*, in vivo and in vitro and on Barley Malt α-amylase in vitro. Kasetsart Journal: Natural Science, 41: 451-460.

Bernfeld, P. 1955. Amylases, α and β. Method Enzyme. 1, 149-158.

Bignell, D. E. and Anderson, J. M. 1980. Determination of pH and oxygen status in the guts of lower and higher termites. Journal of Insect Physiology, 26: 183-188.

De Leo, F., Bonade-Bttino, M., Ceci, L. R., Gallerani, R. and Jouanin, L. 2001. Effects of a mustard trypsin expressed in different plants on three lepidopteran pests. Insect Biochemistry and Molecular Biology, 31: 593-602.

Down, R. E., Fitches, E. C., Wiles, D. P., Corti, P., Bell, H, A. and Gatehouse, J. A. 2006. Insecticidal spider venom toxin fused to snowdrop lectin is toxic to the peach-potato aphid, *Myzus persicae* (Hemiptera: Aphididae) and the rice brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). Pest Management Science, 62: 77-85.

Durgac, C., Ozgen, M., Simek, O., Kacar, Y. A., Kiyga, Y. and Celebi, S. 2008. Molecular and pomological diversity among pomegranate (*Punica granatum* L.) cultivars in eastern Mediterranean region of Turkey. African Journal of Biotechnology, 7: 1294-1301.

Eichenseer, H., Matthews, M. C., Bi, J. L., Murphy, J. B. and Felton, G, W. 1999. Salivary glucose oxidase: multifunctional roles

for *Helicoverpa zea*? Archives of Insect Biochemistry and Physiology, 42: 99-109.

Elpidina, E. N., Vinokurov, K. S., Gromenko, V. A., Rudenskaya, Y. A., Dunaevsky, Y. E. and Zhuzhikov, D. P. 2001. Compartmentalization of proteinases and amylases in *Nauphoeta cinerea* midgut. Arch. Archives of Insect Biochemistry and Physiology, 48: 206-216.

Elzinga, R. J. 1987. Fundamentals of Entomology, 3rd Ed., Prentice Hall, New Jersey.

Falco, M. C. and Silva-Filho, M. C. 2003. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. Plant Physiology and Biochemistry, 41: 761-766.

Fitches, E. C., Bell, H. A., Powell, M. E., Back, E., Sargiotti, C. and Weaver, R. J. 2009. Insecticidal activity of scorpion toxin (ButaIT) and snowdrop lectin (GNA) containing fusion proteins towards pest species of different orders. Pest Management Science, 66: 74-83.

Fitches, E., Edwards, M. G., Mee, C., Grishin, E., Gatehouse, A. M. R. and Edwards, J. P. 2004. Fusion proteins containing insect-specific toxins as pest control agents: snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. Journal of Insect Physiology, 50: 61-71.

Franco, O. L., Melo, F. R., Mendes, P. A., Paes, N. S., Yokoyama, M. and Coutinho, M. V. 2005. Characterization of Two *Acanthoscelides obtectus* a-Amylases and Their Inactivation by Wheat Inhibitors. Journal of Agricultural and Food Chemistry, 53: 1585-1590.

Franco, O. L., Rigden, D. J., Melo, F. R. and Grossi-de-Sa, M. F. 2002. Plant α-amylase. Structure, function and potential for crop protection. European Journal of Biochemistry, 269: 397-412.

Gatehouse, A. M. R., Davidson, G. M., Newell, C. A., Merrywater, A., Hamilton, W. D. O. and Burgess, E. P. J. 1997. Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth room trials. Molecular Breeding, 3: 49-63.

Gatehouse, A. M. R., Norton, E., Davison, G. M., Babbe, S. M., Newell, C. A. and Gatehouse, J. A. 1999. Digestive proteolytic activity in larvae of tomato moth, *Lacanobia oleracea*, Effects of plant protease inhibitors in vitro and in vivo. Journal of Insect Physiology, 45: 545-558.

Hagstrum, D. W. and Subramanyam, B. 2006. Fundamentals in stored-product entomology. AACC International Press, St Paul, Minnesota.

Harrison, R. L. and Bonning, B. C. 2010. Proteases as insecticidal agents. Toxins, 2: 935-953.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. F., Barker, R. F. and Boulter, D. 1987. A novel mechanism of insect resistance engineered into tobacco. Nature, 330: 160-163.

Hosseinkhani, S. and Nemat-Gorgani, M. 2003. Partial unfolding of carbonic anhydrase provides a method for its immobilizationon hydrophobic adsorbents and protects it against irreversible thermoinactivation. Enzyme and Microbial Technology, 33: 179-184.

Ishimoto, M., Sato, T., Chrispeels, M. J. and Kitamura, K. 1996. Bruchid resistance of transgenic azuki bean expressing seed α-amylase inhibitor of common bean. Experimental and Applied Entomology, 79: 309-315.

Isman, M. B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology, 51: 45-66.

Kazzazi, M., Bandani, A. R. and Hosseinkhani, S. 2005. Biochemical characterization of alpha-amylase of the Sunn pest, *Eurygaster integriceps*, Entomological Science, 8: 371-377.

Khan, N. 2011. In vitro Effects of protienaceous alpha amylase inhibitors on red flour Beetle, *Tribolium castaneum*. Science Research Reporter, 1 (2): 101-104.

Kishani-Farahani, H., Goldansaz, S. H. and Sabahi, Q. 2012. A survey on the overwintering larval parasitoids of *Ectomyelois ceratoniae* in three regions in Iran. Crop Protection, 36: 52-57.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature, 227: 680-685.

Madhusudan, Trafny, E. A., Xuong, N. H., Adams, J. A., Ten Eyck, L. F. and Taylor, S. S. 1994. cAMP-dependent protein kinase: crystallographic insights into substrate recognition and phosphotransfer. Protein Science, 3: 176-187.

Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapierre, C. and Hervea du Penhoat, C. L. M. 1997. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. Journal of Agricultural and Food Chemistry, 45 (4): 1195-1201.

Mehrabadi, M., Bandani, A. R. and Kwon, O. 2011. Biochemical characterization of digestive α-d-glucosidase and β-d-glucosidase from labial glands and midgut of wheat bug *Eurygaster maura* (Hemiptera: Scutelleridae). Journal of the Entomological Research Society, 41: 81-87.

Mehrabadi, M., Bandani, A. R., Mehrabadi, R. and Alizadeh, H. 2012. Inhibitory activity of proteinaceous a-amylase inhibitors from Triticale seeds against *Eurygaster integriceps* salivary a-amylases: Interaction of the inhibitors and the insect digestive enzymes. Pesticide Biochemistry and Physiology, 102: 220-228.

Mehrabadi, M., Bandani, A. R. and Saadati, F. 2010. Inhibition of Sunn pest, *Eurygaster integriceps*, α-amylases by α-amylase inhibitors (T-αAI) from Triticale. Journal of Insect Science, 10: 179.

Mehrabadi, M., Franco, O. L. and Bandani, A. R. 2012. Plant Proteinaceous alpha-Amylase and Proteinase Inhibitors and Their Use in Insect Pest Control. In: Bandani, A. R. (Ed.), New Perspectives in Plant Protection, In Tech Publisher.

Melo, F. R., Sales, M. P., Silva, L. S., Franco, O. L., Bloch, J. R. C. and Ary, M. B. 1999. α-Amylase inhibitors from cowpea seeds. Protein and Peptide Letters, 6: 387-392.

Merkx-Jacques, M. and Bede, J. C. 2004. Caterpillar salivary enzymes: "eliciting" a response. Phytoprotection, 85: 33-37.

Merkx-Jacques, M. and Bede, J. C. 2005. Influence of diet on the larval beet armyworm, *Spodoptera exigua*, glucose oxidase activity. Journal of Insect Science, 5(48): 1536-2442.

Morton, R. L., Schroeder, H. E., Bateman, K. S., Chrispeels, M. J., Armstrong, E. and Higgins, T. J. V. 2000. Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. Proceedings of the National Academy USA, 97: 3820-3825.

Mozaffarian, F., Sarafrazi, A. and Nouri Ganbalani, G. 2007. Host plant associated population variation in the carob moth *Ectomyelois ceratoniae* in Iran: A geometric morphometric analysis suggests a nutritional basis. Journal of Insect Science, ISSN: 1536-2442.

Musser, R. O., Hum-Musser, S. M., Ervin, G., Murphy, J. B. and Felton, G. W. 2002. Caterpillar saliva beats plant defences. Nature, 416: 599-600.

Nasrollahi, A. A., Shojai, M. and Ziaii, M. 1998. Large scale production and application of Trichogramma wasps for biological control of pomegranate moth *Ectomyelois ceratoniae* in Yazd province. Processing of 13th Iranian plant protection congress, 1: 167.

Nay, J. E. and Perring, T. M. 2006. Effect of Fruit Moisture Content on Mortality, Development, and Fitness of the Carob Moth (Lepidoptera: Pyralidae). Environmental Entomology, 35: 237-244.

Ni, N., El-Sayed, M. M., Sanghvi, T. and Yalkowsky, S. H. 2000. The effects of NaCl on the solubility of organic compounds in aqueous solution. Journal of Pharmaceutical Science, 89: 1620-1625.

Norouzi, A., talebi, A. A. and Fathipour, Y. 2008. Development and demographic parameters of the carob moth *Apomyelois ceratoniae* on four diet regimes. Bulletin of Insectology, 61: 291-297.

Peiffer, M. and Felton, G. W. 2005. The host plant as a factor in the synthesis and secretion of salivary glucose oxidase in

larval *Helicoverpa zea*. Archives of Insect Biochemistry and Physiology, 58: 106-113.

Ravan, S., Mehrabadi, M. and Bandani, A. R. 2009. Biochemical characterization of digestive amylase of wheat bug, *Eurygaster maura* (Hemiptera: Scutelleridae). African Journal of Biotechnology, 8 (15): 3640-3648.

Saadati, F., Bandani, A. R. and Moslemi, A. 2011. Effect of plant seeds protein extract on the Sunn pest, *Eurygaste integriceps* Puton, growth and development and its gut serine protease activity. African Journal of Biotechnology, 10 (55): 11502-11510.

Shakeri, M. 1993. First report of attack of *Spectrobates ceratoniae* Zell. To figs in Iran. Applied Entomology and Phytopathology, 60: 29.

Shakeri, M. 2004. A review on investigations on pomegranate neck worm in Iran. In: A proceeding on evaluation of finding and current problems associated with *Spectrobates ceratoniae* management in pomegranate. Pp: 18-30. Ministry of Agricultural Jihad, organization of research and education, Yazd agriculture and natural resources research center.

Sharma, H. C. and Ortiz, R. 2000. Transgenics, pest management, and the environment. Current Science, 79: 421-437.

Silva, C. P., Terra, W. R., Xavier-Filho, J., De Sa, M. F. G., Lopes, A. R. and Pontes, E. G. 1999. Digestion in larvae of *Callosobruchus maculates* and *Zabrotes subfasciatus* (Coleoptera: Bruchidae) with emphasis on α-amylases and oligosaccharidases. Insect Biochemistry and Molecular Biology, 29: 355-366.

Silva, C. P., Terra, W. R., Xavier-Filho, J., Grossi de Sa, M. F, Isejima, E. M. and DaMatta, R. A. 2001. Digestion of legume starch granules by larvae of *Zabrotes subfasciatus* (Coleoptera: Bruchidae) and the induction of a-amylases in response to different diets. Insect Biochemistry and Molecular Biology, 31: 41-50.

Silva, J. R. A., Rezende, C. M., Pinto, A. C., Pinheiro, M. L. B, Cordeiro, M. C. and Tamborini, E. 1998. Esteres triterpenicos de *Himatanthus sucuuba* (Spruce) Woodson. Quimica Nova, 21: 702-704.

Sivakumar, S., Mohan, M., Franco, O. L. and Thayumanavan, B. 2006. Inhibition of insect pest α-amylases by little and finger millet inhibitors. Pesticide Biochemistry and Physiology, 85: 155-160.

Svensson, B., Fukuda, K., Nielsen, P. K. and Bonsager, B. C. 2003. Proteinaceous α-amylase inhibitors. Biochimica et Biophysica Acta, 1696: 145-156.

Valencia, A., Bustillo, A. E., Ossa, G. E. and Chrispeels, M. J. 2000. α-amylase of the coffee berry borer (*Hypothenemus hampei*) and their inhibition by two plant amylase inhibitors. Insect Biochemistry and Molecular Biology, 30: 207-213.

Walker, A. J., Ford, L., Majerus, M. E. N., Geoghegan, I. E., Birch, N. and Gatehouse, J. A. 1998. Characterization of the midgut digestive proteinase activity of the two spot ladybird (*Adalia bipunctata* L.) and its sensitivity to proteinase inhibitors. Insect Biochemistry and Molecular Biology, 28: 173-180.

Yezdani, E., Sendi, J. J., Zibaee, A. and Ghadamyari, M. 2010. Enzymatic properties of a-amylase in the midgut and the salivary glands of mulberry moth, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae). Comptes Rendus Biologies, 333: 17-22.

Yildirim, A., Mavi, A. and Kara, A. 2001. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. Journal of Agricultural and Food Chemistry, 49: 4083-4089.

Zeng, F. and Cohen, A. C. 2000. Partial characterization of α-amylase in the salivary glands of *Lygus hesperus* and *L. lineolaris*. Comparative Biochemistry and Physiology, 126B: 9-16.

Zibaee, A., Bandani, A. R., Kafil, M. and Ramzi, S. 2008. Characterization of α-amylase in the midgut and the salivary glands of rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). Journal of Asia-Pacific Entomology, 11: 201-205.

آلفا-آمیلاز و پروتئیناز غدد بزاقی کرم گلوگاه انار (*Ectomyelois ceratoniae*)، وتأثیر عصاره پروتئینی دانه غلات روی فعالیت آن‌ها

احسان برزویی، علی‌رضا بندانی* و سیدحسین گلدانساز

گروه گیاه‌پزشکی، پردیس کشاورزی و منابع طبیعی دانشگاه تهران، کرج، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: abandani@ut.ac.ir
دریافت: ۱۸ اسفند ۱۳۹۱؛ پذیرش ۱۰ اردیبهشت ۱۳۹۲

چکیده: کرم گلوگاه انار یکی از مخرب‌ترین آفات انار و برخی از محصولات دیگر می‌باشد. اقدامات کنترلی مختلف به‌منظور کنترل این آفت تاکنون انجام شده، اما هیچ‌کدام موفقیت‌آمیز نبوده‌اند. بنابراین، در مطالعه حاضر اثر عصاره پروتئینی دانه غلات از جمله سه رقم گندم (ام وی-۱۷، افلاک و زارع) و تریتیکاله روی فعالیت آلفا-آمیلاز و پروتئاز غدد بزاقی این حشره مورد بررسی قرار گرفتند. نتایج حاصل، مهار فعالیت آلفا-آمیلاز را ۳۸، ۴۴، ۲۸ و ۷۶٪ به‌ترتیب در حضور عصاره پروتئینی تریتیکاله، ام وی-۱۷، افلاک و زارع نشان داد. مطالعات بیشتر با رقم گندم زارع انجام شد. استفاده از غلظت‌های مختلف ۱۳، ۶/۵، ۳/۲۵، ۱/۶۲۵ و ۰/۸۱۲۵ میکروگرم پروتئین باعث مهار فعالیت آنزیم آلفا-آمیلاز به‌ترتیب ۷۶، ۷۵، ۶۸، ۶۰ و ۴۲٪ شد. نتایج سنجش در ژل داده‌های اسپکتروفتومتری را تأیید کرد و نشان داد که اثر عصاره بذر روی آنزیم به‌صورت وابسته به دز بود. بررسی تأثیر عصاره پروتئینی بر فعالیت پروتئاز، روند مشابهی را نشان داد. این داده‌ها نشان می‌دهد که گیاهان، پروتئین‌های مختلف با ویژگی‌های متفاوتی تولید می‌کنند که روی آنزیم‌های گوارشی حشرات گیاهخوار تأثیر می‌گذارند و برخی از آن‌ها می‌توانند برای کنترل حشرات در برنامه‌های مدیریت تلفیقی آفات مورد استفاده قرار گیرند.

واژگان کلیدی: کرم گلوگاه انار، آلفا-آمیلاز، پروتئاز، عصاره دانه غلات